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Gating of Retinal Rod Cation Channel by Different Nucleotides: Comparative Study of Unitary Currents

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Summary. Single channels are observed after incorporation of native vesicles from bovine rod outer segment membranes into planar lipid bilayers. The activity of a single channel in the presence of cGMP is compared to that induced by the analog 8-bromo-cGMP and by cAMP. At +80 mV, $K_{0.5}$ is about 3 μM for 8Br-cGMP, 18 μM for cGMP and 740 μM for cAMP. In cAMP, the amplitude of the current is smaller than in cGMP or 8Br-cGMP and depends on the filter cut-off frequency. The open/closed transition rates of the channel are slightly slower with 8Br-cGMP than with cGMP while they are 5 to 10 times faster with cAMP. Addition of Ni^{2+} ions to either cGMP or cAMP increases the open probability: the open/closed transition rates and amplitude of the current in cAMP are then comparable to those in cGMP. A dual effect of the addition of cAMP on the cGMP- or 8Br-cGMP-dependent activity previously reported (Furman, R.E., Tanaka, J.C. 1989. *Biochemistry* **28:**2785–2788) is observed with a single channel: addition of subthreshold cAMP concentrations to cGMP (or to 8Br-cGMP) markedly increases P_o; addition of cAMP concentrations higher than about 70 μM progressively accelerates the kinetics and reduces the amplitude to values observed in cAMP alone. The results are discussed in relation with the model previously proposed to account for the existence of four current levels (Ildefonse, M., Bennett, N. 1991. *J. Membrane Biol.* **123:**133–147).

Key Words vision · phototransduction · nucleotide-gated channels · bilayers

Introduction

Cyclic GMP-dependent cation channels of the plasma membrane of retinal rods, opened in the dark and closed by a light flash, are responsible for the hyperpolarization observed in response to light (*see* Pugh & Cobbs, 1986; Yau & Baylor, 1989; Pugh & Lamb, 1990). Patch-clamp experiments have shown that the channel is directly regulated by cooperative binding of at least three cGMP molecules (Fesenko, Kolesnikov & Lyubarsky, 1985; Zimmerman et al., 1985; Haynes, Kay & Yau, 1986; Zimmerman & Baylor, 1986; Matthews, 1987). The protein corresponding to the cGMP-gated channel has been purified: a 63-kDa polypeptide isolated by Cook et al. (1986) was identified as a functional cGMP-gated channel when reconstituted into phospholipid vesicles (Cook et al., 1986) or into planar bilayers (Hanke, Cook & Kaupp, 1988). The amino acid sequence deduced from the cDNA (Kaupp et al., 1989) predicts a molecular mass of 79.6 kDa. This value is close to the value of 78 kDa found more recently by Hurwitz and Holcombe (1991). A domain situated near the carboxy-terminus of the protein was identified as a single cGMP-binding site (Kaupp et al., 1989; *see also* Kaupp, 1991, for a review on these new results on the cGMP-gated channel); the authors suggested that the functional protein is an oligomer composed of four or five subunits. As pointed out by Jan and Jan (1990), structural similarities exist between the cGMP-gated channel and the voltage-activated potassium channel. Mutagenesis studies have shown that the functional potassium channel is formed by the co-assembly of four subunits arranged in a circular array to form a central cation-conductive pore (reviewed by MacKinnon, 1991): by analogy with this channel, the cGMP-gated channel subunits could be organized in the same way.

The efficiency of other nucleotides for channel opening was previously compared to that of cGMP in studies of macroscopic currents measured from patch-clamp recordings of photoreceptors (Zimmerman et al., 1985; Tanaka, Eccleston & Furman, 1989) or of channels expressed in *Xenopus* oocytes (Altenhofen et al., 1991). In particular, cAMP has been shown to be much less potent than cGMP (Tanaka et al., 1989; Altenhofen et al., 1991). The selectivity of the binding site for cGMP or cAMP seems to be respectively related to the presence of a threonine or alanine residue in the nucleotide site. This was first proposed from a comparative study of nu-

cleotide-binding domains of cGMP- and cAMP-dependent protein kinases (Weber, Shabb & Corbin, 1989) and was recently confirmed for both the rod and olfactory channels from experiments in which the replacement of Thr-560 (rod) or Thr-537 (olfactory) by an Ala residue and subsequent expression of the mutant channels in *Xenopus* oocytes resulted in a 30-fold lower sensitivity to cGMP (Altenhofen et al., 1991). Furman and Tanaka (1989) have also reported an interaction between cAMP and cGMP on photoreceptor channel activation. At saturating concentrations of cGMP and cAMP, the current observed in the presence of the two nucleotides was less than the sum of individual responses, suggesting that the same population of channels was activated by cAMP and cGMP. The authors described a two-phase effect of the combination of cAMP and cGMP, depending on the cAMP concentration: addition of cAMP concentrations lower than the $K_{0.5}$ to a fixed, subsaturating concentration of cGMP increased the macroscopic current; further increase of cAMP concentration reduced the current to the lower current-amplitude plateau produced by cAMP alone. A potentiating effect of cAMP when the channel was opened by 8Br-cGMP was also described by Filatov et al. (1989).

In a previous work (Ildefonse & Bennett, 1991), we have studied single-channel activity after incorporation of native bovine rod membrane vesicles into planar bilayers. The cGMP dependence suggests the existence of four binding sites for cGMP and shows that sequential binding of four cGMP molecules corresponds to the opening of four discrete conductance levels. In the experiments reported here, we compare the characteristics of unitary currents (conductance, open probability and rate constants) in the presence of cGMP, 8Br-cGMP or cAMP, in order to determine at the single-channel level the differences previously observed in patch-clamp experiments on macroscopic currents. The combined effects of cAMP and cGMP (or 8Br-cGMP) on a single channel is also described.

Materials and Methods

Membrane Vesicles Preparation

Bovine rod outer segments were purified as described by Kühn (1984). The membranes were then extensively washed in room light with hypotonic buffer in the presence of GTP in order to remove all soluble proteins as well as the peripheral G-protein and cGMP-phosphodiesterase. After at least four additional hypotonic washes, the membranes were frozen in liquid nitrogen.

Planar Bilayers and Vesicle Incorporation

Phosphatidylethanolamine (PE) and phosphatidylserine (PS) 70:30 (wt/wt), purified from bovine brain (Avanti Polar Lipids, Birmingham, AL) were dissolved in decane (30 mg/ml) and spread on a hole 200 μm in diameter. Fusion of the rod membrane vesicles added in the *cis* chamber was performed in the presence of 500 mM NaCl in the *cis* chamber, 100 mM in the *trans* chamber. The probability of fusion did not seem to be much increased by the presence of divalent cations and "low divalent" solutions (150 μM $CaCl_2$ + 250 μM EGTA, corresponding to 200 nM free $[Ca^{2+}]$ from dissociation constants reported by Sillen & Martell, 1964; contaminant Mg^{2+}, i.e., no $MgCl_2$ added) were used. In experiments with Ni^{2+} ions, 150 μM $NiSO_4$ was added to the *cis* chamber (corresponding to about 0.2 μM free $[Ni^{2+}]$ in the presence of 150 μM Ca^{2+} and 250 μM EGTA, from Sillen & Martell, 1964); a concentation which produced the maximum effect on the channel activity. All solutions were buffered at pH 7.4 with 10 mM HEPES. cGMP (5 to 20 μM) was added to the *cis* chamber together with rod membrane vesicles (30 to 100 μg/ml, less than 1% of the 1.5-ml chamber volume) in order to allow immediate visualization of channel incorporation which usually occurred after a delay of a few minutes to 1 hr. After channel(s) incorporation, the *cis* chamber was perfused one or several times while stirring in order to reduce the membrane and cGMP concentrations and to modify the composition of the solution if required (one perfusion leads to at least 20-fold dilution). The absence of activity was checked before any further nucleotide addition. Washout of the chamber or addition of chemicals did not modify the position of the baseline by more than 0.1 pA, allowing unambiguous recognition of the closed state. The experiments were carried out in room light at 20–22°C.

Recording of Channel Activity and Analysis

Currents were recorded with a Bio-Logic RK-300 patch-clamp amplifier equipped with a 10-GΩ feedback headstage. Bandwidth was 3 kHz. Potential values were defined as *cis* chamber minus *trans* chamber voltages, according to the physiological convention. Amplitude and dwell-time histograms and power spectral densities were built and fitted with the Bio-Patch software (Bio-Logic). Records of 20-sec duration were sampled at 12 kHz and then filtered (Gaussian digital filter) to values stated in the legends of the figures.

Amplitude Histograms

After digital filtering, the minimum duration of events that can be measured with less than 10% reduction of their amplitude is 0.5 msec at 1 kHz, 1.5 msec at 300 Hz and 4.5 msec at 100 Hz. The open probability is defined from the amplitude histograms as $P_o = 1 - P_c$ (with P_c = proportion of the Gaussian curve corresponding to the closed state). Experimental dose-response data were fitted to the Hill equation:

$$P_o = P_{o\,\max}/[1 + (K_{0.5}/L)N_h] \quad (1)$$

with a Levenberg-Marquardt algorithm (MicroCal Origin software), where P_o is the open probability at a given nucleotide concentration L, $P_{o\,\max}$ the open probability at saturation of the nucleotide, $K_{0.5}$ the nucleotide concentration at half-saturation and N_h the index of cooperativity (Hill coefficient).

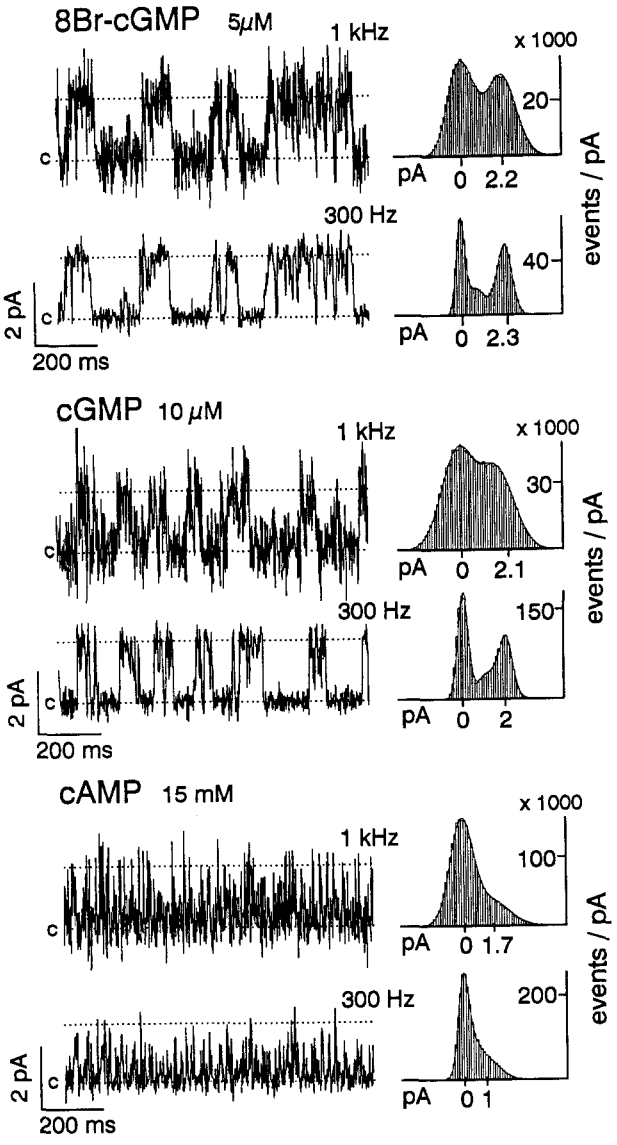

Fig. 1. Comparison of single-channel opening by 8Br-cGMP, cGMP and cAMP: current recordings and corresponding amplitude histograms (built from records of 20-sec duration, 1 kHz and 300 Hz). Experiments were carried out in 100 mM NaCl *cis/trans*, "low divalents," at $V = +80$ mV. Notice that the mean level of the cAMP-induced current depends on the filtering conditions: it is reduced by about 40% between 1 kHz and 300 Hz. In all the records, the closed (*c*) and maximum open current levels are underlined.

Dwell-Time Histograms

The threshold used to discriminate between closed and open states was taken at half-amplitude between the closed-state Gaussian curve and the first open-state Gaussian curve. With this standard detection method, using a 300-Hz cut-off frequency Gaussian filter, dwell times longer than 1 msec can be measured accurately.

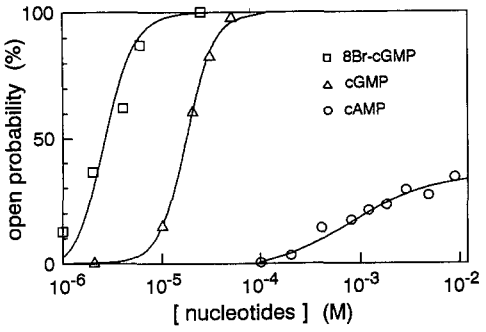

Fig. 2. Open probability of a single channel as a function of 8Br-cGMP, cGMP and cAMP concentrations (same conditions as in Fig. 1). The solid curves are obtained after fitting the experimental points with the Hill equation. For cGMP, with $P_{o\,max}$ fixed at 100%, the best fit is obtained with $K_{0.5} = 17.7 \pm 0.5$ μM and $N_h = 3.1 \pm 0.2$. With the same values for $P_{o\,max}$ and N_h, a $K_{0.5}$ of 2.9 ± 0.3 μM is obtained for 8Br-cGMP. For cAMP, the data cannot be fitted with $N_h = 3.1$; the best fit is obtained with $P_{o\,max} = 43 \pm 4\%$, $K_{0.5} = 743 \pm 200$ μM and $N_h = 1.1 \pm 0.3$.

Power Spectral Densities

Power spectral densities were performed from records of about 60 sec sampled at 12 kHz.

Chemicals

8Br-cGMP, cGMP and cAMP were purchased from Sigma. A possible contamination of cAMP by cGMP was tested by comparision of elution profiles of cGMP and cAMP obtained with anion-exchange chromatography (FPLC, Pharmacia PF SAX column, NaCl gradient, pH8): cGMP, if present in cAMP, would be less than 0.05%. Using cAMP from the same origin, Tanaka et al. (1989) estimated cGMP contamination as less than 0.02%.

Results

Nucleotide Dependence of the Activity of a Single Channel

Conductance and Open Probability

Records in Fig. 1 illustrate, at the same time scale and same potential, the activity of a single channel observed in the presence of 8Br-cGMP (upper panel), cGMP (middle panel) or cAMP (lower panel) after digital filtering at either 1 kHz or 300 Hz. The open/closed transitions are much faster in the presence of cAMP than in the presence of cGMP or 8Br-cGMP. Furthermore, the amplitude histograms show that the amplitude of the current filtered at 1 kHz is smaller in cAMP than in 8Br-cGMP or in cGMP and is reduced by about 40% with further filtering at 300 Hz. In 8Br-cGMP or in cGMP, the

maximum level of current is almost the same and corresponds to a conductance of 25–28 pS; as previously described (Ildefonse & Bennett, 1991), it is not modified between 1 kHz and 300 Hz.

In the presence of millimolar concentrations of cAMP, the open probability of the channel is low (P_o = 33%, corresponding to the maximal open probability in this experiment) compared to those observed with micromolar concentrations of cGMP (P_o = 46%) or of the most efficient nucleotide 8Br-cGMP (P_o = 61%).

The nucleotide dependence of the open probability is illustrated in Fig. 2 from three similar experiments in which the activity of a single channel was tested as a function of the nucleotide concentration. The experimental points were fitted with the Hill equation (Eq. (1), in Materials and Methods) which provides a convenient tool for a comparative study of the different nucleotide-dependent activities. The values of 3.1 for N_h and 17.7 μM for $K_{0.5}$ obtained from the fit of the experimental points in cGMP are in good agreement with the values previously found (Ildefonse & Bennett, 1991) and in favor of the existence of four sites for cGMP as proposed. For simplicity, we have chosen to fix the Hill coefficient to this value of 3.1 for all experiments described below, unless otherwise specified. The observation of four current sublevels when the channel is opened by 8Br-cGMP (Ildefonse & Bennett, 1991) justifies this choice: with N_h = 3.1, a $K_{0.5}$ around 3 μM is obtained, comparable to the value of 1.7 μM given by Zimmerman et al. (1985) for macroscopic current. With cAMP, the data cannot be fitted with N_h = 3.1; the values of the parameters that give the best fit (43% for $P_{o\,max}$, 743 μM for $K_{0.5}$ and 1.1 for N_h) are comparable to those given by Tanaka et al. (1989) for multichannel currents and confirm that the apparent affinity of the channel for cAMP is much lower than for cGMP and 8Br-cGMP.

Kinetics

Dwell-time histograms and their corresponding fits from experiments with 2 μM 8Br-cGMP (P_o = 36%), 20 μM cGMP (P_o = 56%) and 10 mM cAMP (P_o = 40%) are shown in Fig. 3*A*. In the presence of 8Br-cGMP or cGMP, the lifetime distributions of the closed and open states are fitted with two exponentials. The time constants of the fast components lie between 0.2 and 1.7 msec, close to the limit of the filter and may include noise transitions. For this reason, only the values of the slow components, referred to as τ_c for the closed state and τ_o for the open state, are given in the dwell-time histograms (τ_c = 59 msec, τ_o = 20 msec in 8Br-cGMP; τ_c = 16 msec, τ_o = 12 msec in cGMP). In the presence of cAMP, the duration histograms in the two states are described by a single exponential with time constants much shorter than those of the slow components in cGMP or 8Br-cGMP (τ_c = 2.3 msec, τ_o = 2.7 msec).

In order to compare the values of the rate constants measured in 8Br-cGMP, cGMP and cAMP under conditions of similar channel activity, τ_c and τ_o were plotted as a function of the open probability P_o (Fig. 3*B*). In the stationary regime, for a particular value of the nucleotide concentration, and therefore of P_o, τ_o and τ_c are related quantities. Since, when τ_c (or τ_o) is plotted as a function of P_o, τ_o (or τ_c) also varies, no attempt was made to fit the curves. At a given P_o, the transition rates are slower for 8Br-cGMP than for cGMP and much faster for cAMP. With 8Br-cGMP or cGMP, the relative decrease of τ_c with increasing open probability (from about 100 to 10 msec in 8Br-cGMP and 100 to 4 msec in cGMP) seems to be more important than the relative increase in τ_o (from about 10 to 50 msec in 8Br-cGMP and 4 to 30 msec in cGMP), in agreement with the previous results of Hanke et al. (1988). Although it is not clearly visible on Fig. 3*B* because of the time scale, when the channels are opened with cAMP, the mean closed and open times also depend on the nucleotide concentration: τ_c is shortened from around 20 msec near the threshold cAMP concentrations (100–200 μM) to about 2.5 msec at saturation (starting around 3 mM); in the same conditions, τ_o is increased from 1 to about 2.7 msec.

In order to determine whether the fast components observed in the presence of 8Br-cGMP or cGMP are mostly due to noise transitions or also represent fast open/closed channel transitions, we performed power spectral analyses for the same currents as analyzed in Fig. 3 but before digital filtering, i.e., recorded at 3 kHz. With this method of analysis, the contribution of white noise to the spectrum is only a constant additive term that can be cancelled out during the fitting procedure. In reality, due to the presence of a large bilayer capacitance, the white noise spectrum increases for frequencies higher than about 100 Hz: its contribution was eliminated by subtracting the power spectrum of the background current observed without any nucleotide (*b* curves in Fig. 4*A*) from that of the current in the presence of a nucleotide (*a* curves in Fig. 4*A*). The spectral densities thus obtained with 8Br-cGMP, cGMP and cAMP are shown in Fig. 4*B*. In the presence of either 8Br-cGMP or cGMP, two Lorentzian functions are necessary to fit the power spectra, suggesting that the channel activity involves both a fast and a slow mechanism. In the presence of cAMP, the power spectrum can be fitted by a single Lorentzian. The

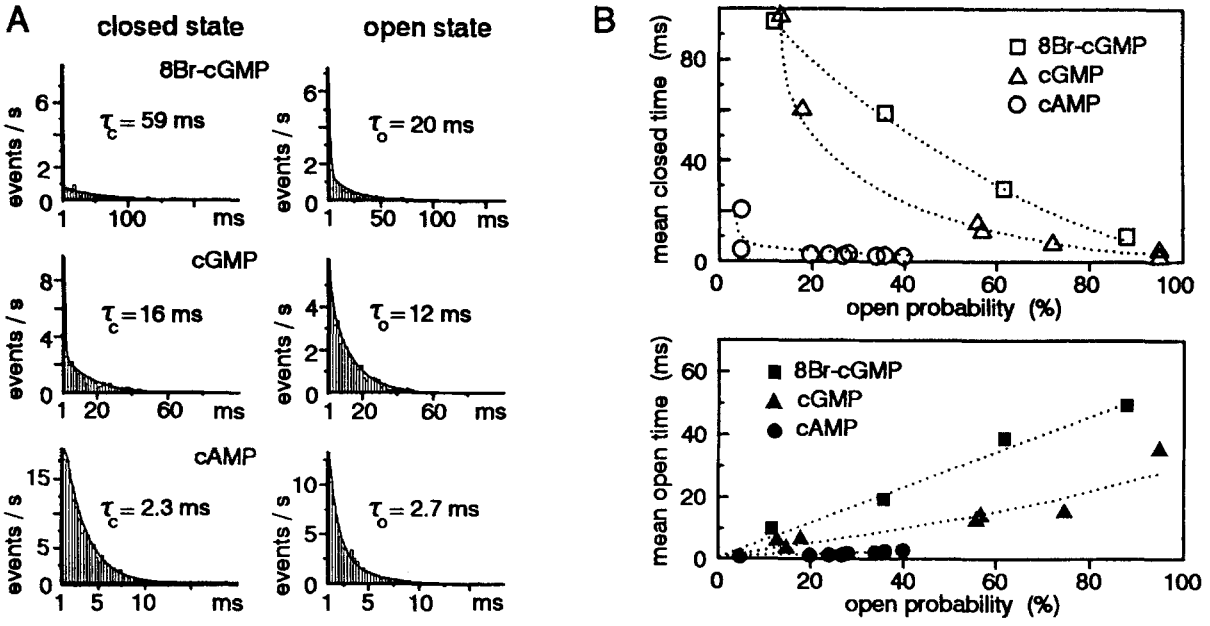

Fig. 3. Kinetic analysis of a single channel in the presence of 8Br-cGMP, cGMP and cAMP (same experimental conditions as in Fig. 1; 300 Hz). (*A*) Duration histograms built from records of 20 sec. The open probabilities are 36% with 2 μM 8Br-cGMP, 56% with 20 μM cGMP and 40% with 10 mM cAMP. When the channel is opened with either 8Br-cGMP or cGMP, two components are detected in both closed and open states: the time constant for the slow component is indicated on the histogram; time constants between 0.2 and 1.7 msec are measured for the fast component. (*B*) Dependence of the mean closed time τ_c and of the mean open time τ_o (slow components for 8Br-cGMP and cGMP) on the open probability (concentrations from 1 to 6 μM 8Br-cGMP, from 5 to 30 μM cGMP, and from 200 μM to 9 mM cAMP). Notice the small values obtained in cAMP compared to those in 8Br-cGMP and cGMP and that the kinetics in 8Br-cGMP are slightly slower than in cGMP, as can be observed on the records of Fig. 1.

relaxation times τ_r calculated from the corner frequencies ($\tau_r = 1/2\pi Fc$) depend on the nucleotide in the same way as the mean closed and open times τ_c and τ_o determined from duration histograms in Fig. 3. With all three nucleotides, the values obtained from the two kinds of analyses for the slower components are in satisfactory agreement with the simplified two-state relation:

$$1/\tau_{r\,\text{slow}} = 1/\tau_c + 1/\tau_o. \quad (2)$$

Agreement with this relation is not so good for the fast components ($\tau_{r\,\text{fast}}$, $\tau_{o\,\text{fast}}$ and $\tau_{c\,\text{fast}}$). At least two reasons can explain the difference: (i) we have very little confidence in the values of $\tau_{o\,\text{fast}}$ and $\tau_{c\,\text{fast}}$ because of noise transitions and (ii) the simple two-state model cannot be used for the fast components since they cannot be studied independently of the slow components in our experimental conditions. Nevertheless, the important point that can be deduced from the spectral density analysis is that $\tau_{o\,\text{fast}}$ and $\tau_{c\,\text{fast}}$, although inaccurate, are really associated with fast open/closed channel transitions.

Effects of Ni^{2+} on the Activity of a Single Channel in the Presence of cGMP or of cAMP

In a previous work (Ildefonse & Bennett, 1991), we described a potentiating effect on channel activity of several divalent cations (Co^{2+}, Ni^{2+} and Fe^{2+}) at the cytoplasmic side. The open probability was largely increased in the presence of Co^{2+}, with a visible increase in the mean open time. We have further studied and compared the effect of Ni^{2+} ions when the channel is opened by cGMP or cAMP.

The effects of Ni^{2+} ions on channel activity in the presence of either cGMP or cAMP are illustrated in Fig. 5*A* and *B*. With each nucleotide, the records and corresponding amplitude histograms were obtained from experiments with the same channel before and after addition of Ni^{2+}. In cGMP, as well as in cAMP, the open probability is spectacularly increased by the addition of Ni^{2+} (from 13 to 93% in cGMP and from 6 to 65% in cAMP), in relation with a large increase in the mean open time, as previously shown with Co^{2+} ions. In the experiment with cAMP (Fig. 5*B*), the addition of Ni^{2+} ions reveals the pres-

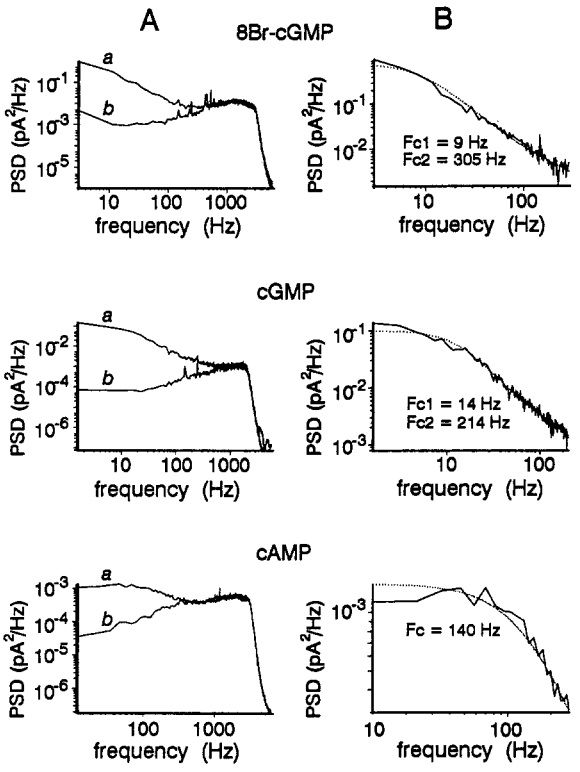

Fig. 4. Power spectral densities (PSD) of a single channel in the presence of 8Br-cGMP, cGMP and cAMP (same experiments as in Fig. 3, no digital filtering). (*A*) The *a* curves correspond to PSD in the presence of the nucleotide; the *b* curves correspond to PSD in the same bilayer without the nucleotide (or with a very low concentration in the case of 8Br-cGMP). (*B*) Power spectral densities of single-channel activity (*a–b*). In the presence of cAMP, the PDS can be fitted by a single Lorentzian with a corner frequency corresponding to a relaxation time τ_r of 1.13 msec ($2\pi Fc = 1/\tau_r$). In the presence of 8Br-cGMP or of cGMP, two Lorentzian functions are needed to fit the power spectrum of the working channel, with corner frequencies corresponding to relaxation times τ_r = 18 and 0.5 msec in 8Br-cGMP and τ_r = 11.4 and 0.74 msec in cGMP.

ence of two channels in the bilayer. Moreover, the amplitude of the current reached by one channel when opened by cAMP in the presence of Ni^{2+} is the same as the amplitude measured in cGMP. In another experiment with only one channel (Fig. 6), the four sublevels previously described with cGMP can be observed in the presence of cAMP + Ni^{2+} ions. The effects of Ni^{2+} ions on the dependence of the open probability of a single channel upon the nucleotide concentration is shown in the lower panels of Fig. 5: in the presence of cGMP (Fig. 5*A*), $K_{0.5}$ is shifted from 16 to 4 μM by the addition of Ni^{2+}. Since four sublevels were observed in cAMP + Ni^{2+}, N_h = 3.1 was taken to fit the experimental values (Fig. 5*B*): $K_{0.5}$ is reduced from 740 to 225 μM in the presence of Ni^{2+} and $P_{o\,max}$ is increased from 43 to 72%.

Figures 7 and 8 illustrate the kinetic analysis of the experiments shown in Fig. 5. Dwell-time histograms at a given cGMP concentration (Fig. 7) show that the mean closed time τ_c is drastically reduced (from 97 to 7.4 msec in Fig. 7*A*) and the mean open time τ_o increased (from 7.8 to 59 msec in Fig. 7*B*) in the presence of Ni^{2+}. However, when related to the open probability (lower panels of Fig. 7), the mean open and closed times are not modified in the presence of Ni^{2+} ions. When the channel is opened by cAMP (Fig. 8), the same effects of Ni^{2+} ions on the kinetics are observed, i.e., a decrease of τ_c (from 22 to 7 msec in Fig. 8*A*) and a very large increase of τ_o (from 0.6 to 11 msec in Fig. 8*B*). It must also be noted that in the presence of cAMP + Ni^{2+}, the dwell-time histograms for the two states reveal fast components (between 0.2 and 1.5 msec) comparable to those observed in Fig. 3 with cGMP or 8Br-cGMP and the power spectral density of current records in the presence of cAMP + Ni^{2+} are also fitted by two Lorentzians (*not shown*). When related to the open probability of the channel (lower panels), both the mean open and closed times τ_o and τ_c are clearly increased by the addition of Ni^{2+}: the maximum value of τ_o in cAMP + Ni^{2+} is about five times that in cAMP alone at saturation (Fig. 8*B*). The values of τ_c and τ_o as a function of the open probability in cAMP + Ni^{2+} approach those shown for cGMP in Fig. 3 or for cGMP + Ni^{2+} in Fig. 7*B*.

We have previously reported (Ildefonse & Bennett, 1991) a well-marked outward rectification of the conductance of a single channel, even with low divalent concentration. Haynes et al. (1986) measured shorter open times for negative voltages, and the records shown in both our work and the work of Hanke et al. (1988) suggested faster kinetics for the inward current. This property, together with the limitation due to our experimental conditions which prevent an accurate measurement of fast kinetics (*see* Materials and Methods) could result in underestimating the inward current and therefore account for the rectification observed. We measured the current induced by each of the three nucleotides as a function of voltage in the presence of Ni^{2+} ions which drastically increase the mean open time. The current-voltage relation in Fig. 9 shows that, in the presence of Ni^{2+}, the conductance of the channel and its voltage dependence are the same with 8Br-cGMP, cGMP or cAMP and that the outward rectification is still observed. The conductance at negative potentials is 20 pS, slightly higher than the previously reported value of 16 pS, suggesting that in the absence of Ni^{2+}, the amplitude of the currents is indeed slightly underestimated. With all three nucleotides, the addition of Ni^{2+} did not qualitatively modify the dependence of the open probability on

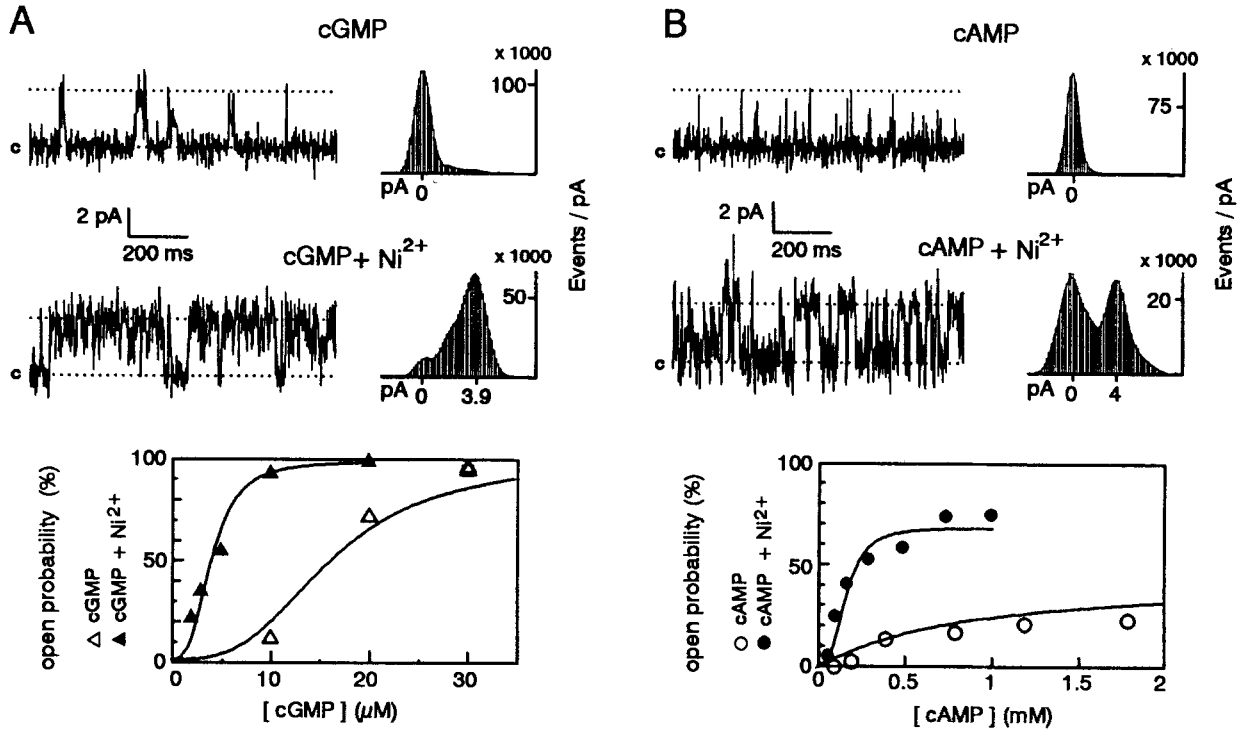

Fig. 5. Potentiating effect of Ni^{2+} on open probability of channels observed in the presence of cGMP (*A*) or cAMP (*B*). In the two experiments, the conditions were NaCl 500 mM *cis*/100 mM *trans*, "low divalents," $V = +60$ mV, 150 μM $NiSO_4$. The records and corresponding amplitude histograms (built from records of 20-sec duration; 300 Hz) in the top panels illustrate the effect of Ni^{2+} with 10 μM cGMP in *A*, with 300 μM cAMP in *B*: the open probability is increased from 13 to 93% in cGMP and from 6 to 65% in cAMP. In cAMP, the maximum amplitude of the current in the presence of Ni^{2+} is the same as with cGMP. In all the records, the closed (*c*) and maximum open current levels are underlined. Dose-response relations with or without Ni^{2+} ions shown in the bottom panels were obtained from the same experiment for cGMP and from two different experiments for cAMP (one in cAMP alone, another one in cAMP + Ni^{2+} because it is difficult to achieve a correct washing of the high cAMP concentration needed to reach the maximum P_o). Experimental data were fitted with the Hill equation (solid lines): for cGMP, with the same $P_{o\,max}$ (100%) and N_h (3.1), $K_{0.5}$ is shifted from 16 ± 0.7 μM to 4 ± 0.3 μM by Ni^{2+}; for cAMP, the reference experiment is the same as that illustrated in Fig. 1 (first points and beginning of the fit with $P_{o\,max} = 43\%$, $K_{0.5} = 743$ μM and $N_h = 1.1$). After addition of Ni^{2+}, experimental points were fitted with N_h fixed at 3.1: in this case, values obtained for $P_{o\,max}$ and $K_{0.5}$ are 72 ± 3% and 225 ± 22 μM, respectively.

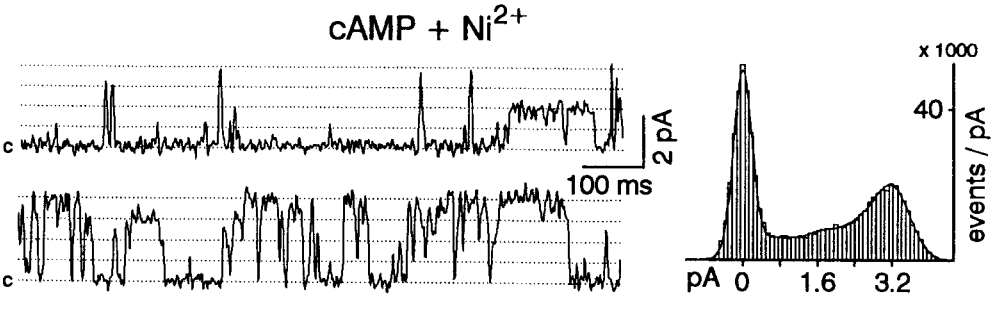

Fig. 6. Resolution of four sublevels in the presence of 300 μM cAMP and Ni^{2+} ions (same conditions as in Fig. 5, but filtered at 100 Hz). Two parts of the 20-sec record used to build the amplitude histogram are shown on the left: in the upper trace, mainly the first and the second sublevels are observed while the third and fourth levels are also reached in the lower trace (the closed (*c*) and the four open current levels are underlined). The fit of the amplitude histogram is calculated with five Gaussian curves, with the intermediate current amplitudes being fixed at 25, 50 and 75% of the maximum value; the total P_o is 62%. Notice that the mean dwell times measured at this P_o in Fig. 8 (τ_c and τ_o around 10 msec) are higher than the minimum dwell time defined by the 100-Hz cut-off filter, which allows measurement of 90% of the current amplitude (4.5 msec).

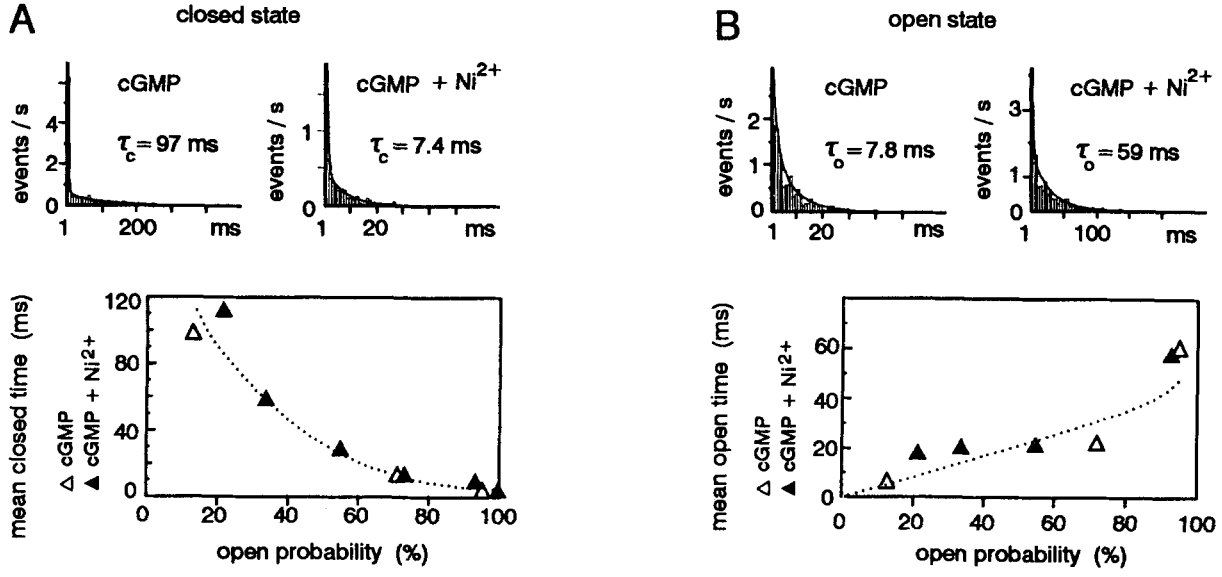

Fig. 7. Effects of the addition of Ni^{2+} to cGMP on the mean closed and open times τ_c (*A*) and τ_o (*B*) (slow components; same experiment and conditions of analysis as in Fig. 5). For the duration histograms illustrated in the top panels, the cGMP concentration was 10 μM, corresponding to a P_o of 13%: at a given cGMP concentration, τ_c is reduced and τ_o is increased by the addition of Ni^{2+}, consistent with the larger P_o observed (93%). In the lower panels, relations between τ_c or τ_o and P_o (concentrations from 10 to 30 μM in cGMP, from 2 to 20 μM in cGMP + Ni^{2+}) show that, at a given open probability P_o, the slow components of mean open and closed times (τ_o and τ_c) are the same with or without Ni^{2+}.

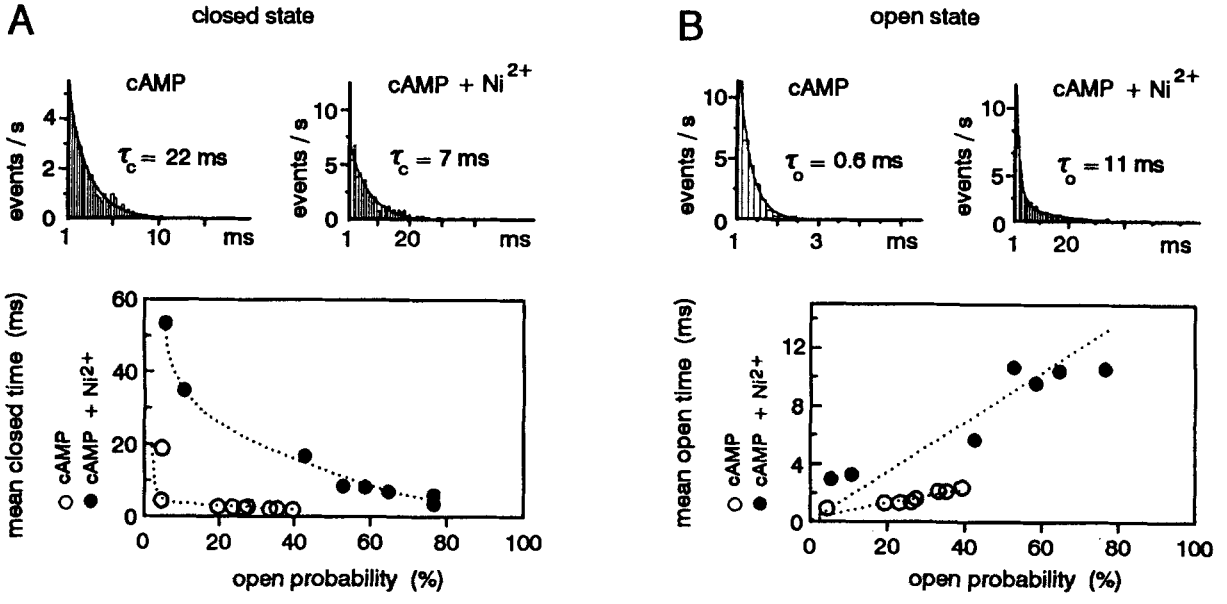

Fig. 8. Effects of the addition of Ni^{2+} to cAMP on the mean closed time τ_c (*A*) and the mean open time τ_o (*B*) (same conditions as in Fig. 5). Similar to the results obtained with cGMP, at a given cAMP concentration (300 μM cAMP for the dwell-time histograms of the top panels, corresponding to a P_o of about 6%), τ_c is reduced and τ_o is increased by the addition of Ni^{2+}, consistent with the larger P_o (65%) observed. Besides, the fit of the dwell-time histograms after the addition of Ni^{2+} ions reveals the presence of a fast component (between 0.2 and 1.5 msec) as already observed with cGMP or with 8Br-cGMP alone. The relation between τ_c or τ_o (slow components for cAMP + Ni^{2+}) and P_o (concentrations from 200 μM to 9 mM in cAMP and from 20 to 200 μM in cAMP + Ni^{2+}) shown in the lower panels (same experiment as in Fig. 1 for cAMP alone and as in Fig. 5 in the presence of Ni^{2+}) indicates that, at a given open probability P_o, the mean open and closed times τ_o and τ_c are longer in the presence of Ni^{2+}.

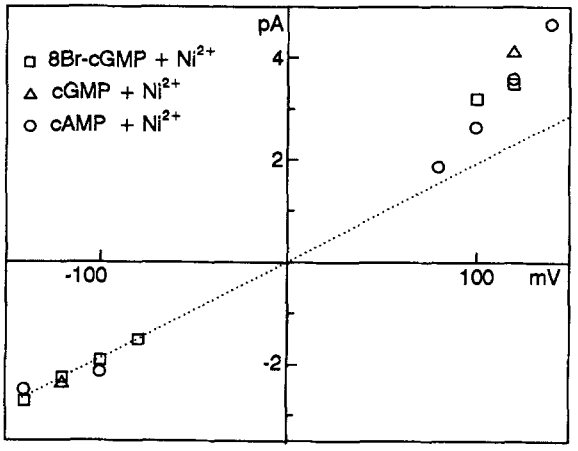

Fig. 9. Current (maximum level)-voltage relations for a single channel opened by 8Br-cGMP, cGMP or cAMP in the presence of Ni^{2+}. Experiments were carried out in 100 mM NaCl *cis/trans*, "low divalents." Notice that the conductance is the same with the three nucleotides and that outward rectification is observed.

the voltage: P_o remained lower for negative potentials than for positive ones (*not shown*).

Combined Effects of cAMP and cGMP (or 8Br-cGMP) on Channel Activity

In relation with the works of Furman and Tanaka (1989) and of Filatov et al. (1989), experiments on the combined effects of cAMP and cGMP (or 8Br-cGMP) on the activity of a single channel are reported in this section.

The records and corresponding histograms in Fig.10 show that the addition of only 30 μM cAMP (a concentration which is far below the threshold concentration observed for the channel activation by cAMP alone) to 15 μM cGMP induces a very large increase in the open probability, also revealing the presence of a second channel in the bilayer. Dose-response curves for cGMP and cGMP + cAMP drawn from another experiment with a single channel are shown below. Fitting the experimental data with the Hill equation and with the same $N_h = 3.1$ for the two curves leads to a shift in $K_{0.5}$ from 12 to 6 μM.

In Fig. 11, dwell-time histograms corresponding to the activity of a channel induced by cGMP alone or by the mixture of the two nucleotides show that the mean closed time is reduced (from 60 to 10 msec in Fig. 11*A*) and the mean open time is increased (from 6 to 18 msec in Fig. 11*B*) by the addition of subthreshold cAMP concentrations. When related to the open probability, the kinetic parameters have the same value in cGMP or in cGMP + cAMP (lower panels in Fig. 11*A* and *B*). These results can be

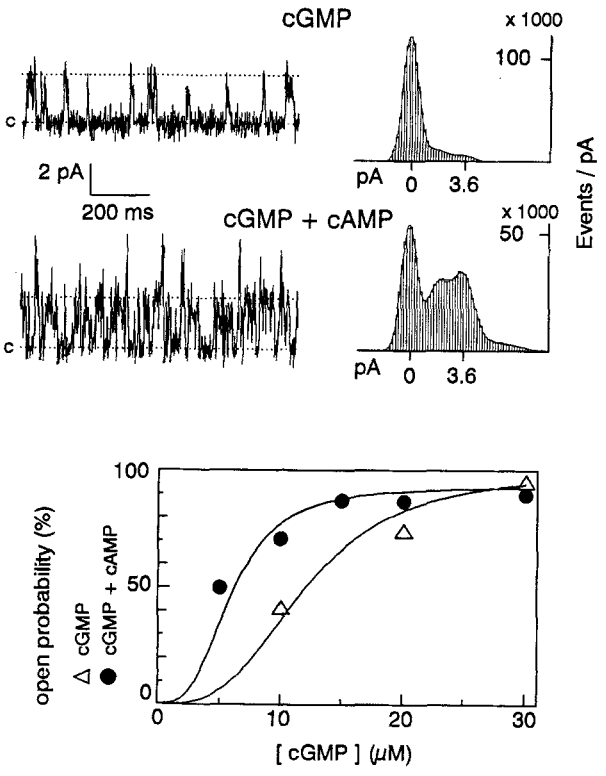

Fig. 10. Potentiating effect of low concentration of cAMP on cGMP-induced activity. The records and corresponding histograms (built from records of 20-sec duration; 300 Hz) illustrate the effect of the addition of 30 μM cAMP to 15 μM cGMP: two channels were incorporated in this experiment (the closed (*c*) and maximum open current levels for a single channel are underlined). The open probability for the two channels is increased from 18 to 64% after addition of cAMP. The dose-response curves obtained in another experiment in which a single channel was expressed show that the presence of 50 μM cAMP increases the sensitivity to cGMP. Fitting the experimental data with N_h fixed at 3.1 leads to a shift of $K_{0.5}$ from 11.7 ± 1.2 μM in cGMP to 6 ± 0.4 μM in cGMP + cAMP. For the two experiments, the conditions were: NaCl 500 mM *cis*/100 mM *trans*, "low divalents"; $V = +60$ mV.

compared to those obtained in the presence of cGMP alone and cGMP + Ni^{2+} ions.

Increasing the cAMP concentration to the millimolar range produces a progressive reduction of the mean unitary current amplitude. The same potentiating and inhibiting effects of cAMP as a function of its concentration are obtained when the channel is opened by 8Br-cGMP (Fig. 12). Addition of only 6 μM cAMP to 1 μM 8Br-cGMP slightly increases the open probability; with increasing cAMP concentration until values are in the millimolar range, the open probability is further increased, but from about 70 μM cAMP, the probability of opening at the maximum 8Br-cGMP-dependent level (2.3 pA) is decreased. When 3 mM cAMP is added to 8Br-cGMP, the value of the mean current is only 1.3 pA, consis-

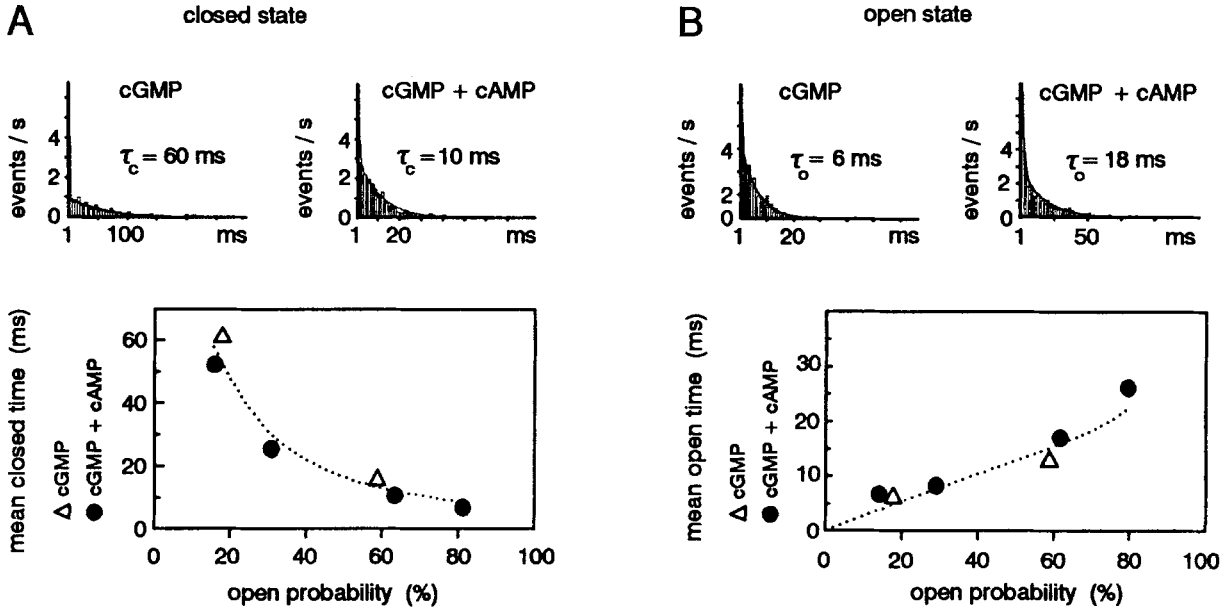

Fig. 11. Effect of the addition of 30 μM cAMP to cGMP on the distribution of closed time (*A*) and of open time (*B*) (same experiment as illustrated in records and amplitude histograms of Fig. 10 from records of 20-sec duration; 300 Hz). The dwell-time histograms in the upper panels illustrate the effect of cAMP in the presence of 15 μM cGMP. P_o is 18% in GMP and 64% in cGMP + cAMP. The relation between the mean closed and open times τ_c and τ_o and open probability P_o (concentrations of 15 and 20 μM in cGMP and from 5 to 20 μM in cGMP + cAMP), in the lower panels, shows that the slow components of the mean open and closed times τ_o and τ_c at a given P_o are the same in cGMP and in cGMP + cAMP.

tent with the value of the current illustrated in Fig. 1 (15 mM cAMP).

Discussion

We report here a comparative study of the gating of a single channel as a function of the nucleotide. The 8Br-cGMP, cGMP and cAMP concentration dependence of the current amplitude, of the open probability and of the kinetics of the channel are described. The potentiating effect of some divalent cations on the opening of the channel, previously illustrated with Co^{2+} ions (Ildefonse & Bennett, 1991) is further studied with Ni^{2+} ions in the presence of either cGMP or cAMP. The competition between cGMP and cAMP previously reported in patch-clamp experiments on macroscopic currents (Filatov et al., 1989; Furman & Tanaka, 1989) is studied in the case of a unitary current.

Conductance, Open Probability and Kinetics of the Channel

The nucleotide concentration dependence of the open probability P_o of a single channel of native bovine rod outer segment membranes in the presence of 8Br-cGMP, cGMP or cAMP is quite comparable to that previously described from macroscopic currents recorded in patch-clamp experiments (Zimmerman et al., 1985; Tanaka et al., 1989; Altenhofen et al., 1991). As previously reported in these studies and in our reconstitution experiments (Ildefonse & Bennett, 1991), the $K_{0.5}$ showed some variability from one experiment to the other, but 8Br-cGMP was always the most efficient, while the affinity of the site for cAMP seemed very low. According to Zimmerman et al. (1985), the higher efficiency of 8Br-cGMP would result from the fact that Br in the 8 position favors the *syn* conformation of the nucleotide, a conformation which would be bound by the channel.

A striking difference between the unitary currents induced by the three nucleotides: 8Br-cGMP, cGMP or cAMP, is the open/closed transition rates. When the channel is opened by either 8Br-cGMP or cGMP, the current reaches a well-resolved amplitude and stays in the closed and in the open state during tens of milliseconds for nucleotide concentrations around $K_{0.5}$. With cAMP, the current is bursting between short-lived closed and open states; the amplitude of the current rarely reaches the maximum level underlined in cGMP or 8Br-cGMP. The smaller amplitude of the current in cAMP could reflect a smaller conductance of the channel opened by this nucleotide or a larger proportion of the current

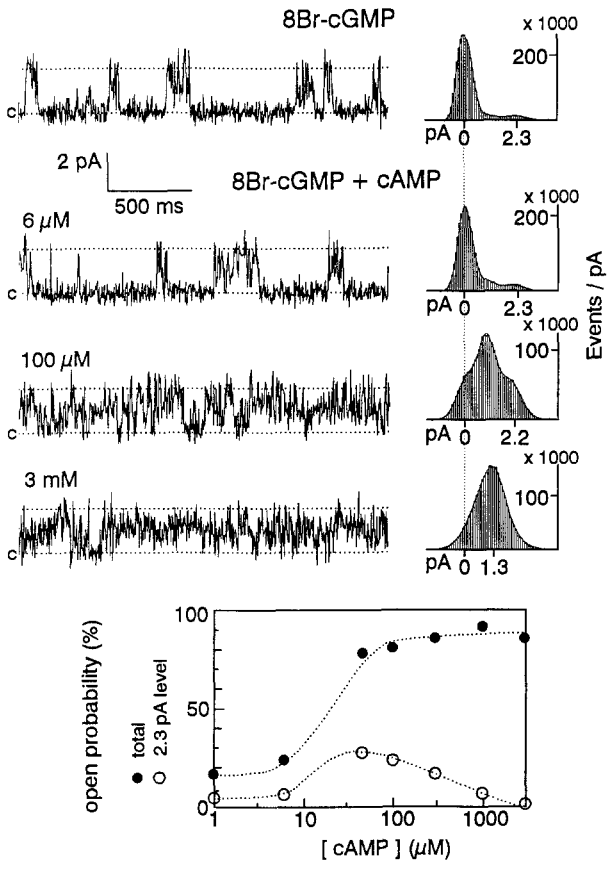

Fig. 12. Dual effect of the addition of cAMP on the opening of a single channel by 1 μM 8Br-cGMP. Records (in 100 mM NaCl *cis/trans*, "low divalents," at $V = +80$ mV) and corresponding amplitude histograms (built from records of 20 sec duration; 300 Hz) illustrate the effect of cAMP at three concentrations: with 6 and 100 μM cAMP, the open probability is increased (from 10 to 17 and 57%, respectively); with 3 mM cAMP, the open probability is further increased to 84%, but the maximum current level observed in 8Br-cGMP alone (underlined together with the closed level *c*) is rarely reached and the mean current amplitude is smaller. In the amplitude histograms, the position of the closed state is underlined for clarity. The relation between cAMP concentration and total open probability P_o(●) or open probability of the 2.3-pA current level (*o*, corresponding to the value of the maximum 8Br-cGMP-induced current) from the same experiment is shown in the lower panel.

sublevels observed in cGMP. The filter cut-off frequency dependence of the amplitude of the current in cAMP also suggests that some fast events might be cut off: thus, the channel may open to the same maximum level as in cGMP but for such short times that it cannot be recorded with its full amplitude, even at 1 kHz. For the same reason, fast events that would occur for subthreshold cAMP concentrations might be undetected in our conditions and the actual position of the dose-response curve in cAMP could be somewhat closer to that in cGMP. In a recent short report from patch-clamp experiments on toad or frog rods, Dhallan, Haynes and Yau (1990) detected single-channel activity with either 1 μM cGMP or 30–100 μM cAMP, corresponding to a conductance of the same value of 25 pS; although no precision is given concerning their recording conditions, the fact that the amplitude of the current is the same in cGMP and cAMP and that the cAMP threshold concentration is lower than in our experiments probably result from a better time resolution of the current records in their experiments.

The scheme that we proposed (Ildefonse & Bennett, 1991) to account for the existence of four current levels as a function of the cGMP concentration, at a given voltage, is represented below.

Scheme *a*

$$\begin{array}{ccccccc} O & \overset{cGMP}{\rightleftharpoons}_{K'1} & O(cGMP) & \overset{cGMP}{\rightleftharpoons}_{K'2} & O(cGMP)_2 & \overset{cGMP}{\rightleftharpoons}_{K'3} & O(cGMP)_3 \overset{cGMP}{\rightleftharpoons}_{K'4} O(cGMP)_4 \\ \updownarrow & & \updownarrow K_{(V_i)^1} & & \updownarrow K_{(V_i)^2} & & \updownarrow K_{(V_i)^3} \qquad \updownarrow K_{(V_i)^4} \\ C & \overset{cGMP}{\rightleftharpoons}_{K1} & C(cGMP) & \overset{cGMP}{\rightleftharpoons}_{K2} & C(cGMP)_2 & \overset{cGMP}{\rightleftharpoons}_{K3} & C(cGMP)_3 \overset{cGMP}{\rightleftharpoons}_{K4} C(cGMP)_4 \end{array}$$

Consistent with the above scheme, three current sublevels can also be observed between the closed state and the maximum current when Ni^{2+} is added to cAMP (Fig. 6). However, it is difficult to know if, in the absence of Ni^{2+}, the proportion of the first sublevels is predominent even at saturating cAMP concentrations, a situation that, as mentioned above, could also account for the smaller amplitude of the current even at 1-kHz cut-off frequency: indeed the Hill coefficient N_h obtained in cAMP is only 1.1, possibly suggesting that only two sites are involved. A more accurate analysis of the current sublevels is needed to test this hypothesis, which would require, however, a more resolutive technique than the one used in the present experiments. It should also be noted that the Hill equation is only a rough approximation of the mechanism of channel opening since it gives a single constant ($K_{0.5}$) for the four steps. Moreover, only the channels with 4 nucleotides bound are assumed to be open in the Hill equation, while those with 1, 2 or 3 nucleotides bound are neglected and only those with no nucleotide bound assumed to be closed. In scheme *a*, each of the four steps (corresponding to the opening of a sublevel) in fact consists of three equilibria: binding of the nucleotide to the closed state, binding to the open state, and the open/closed transition. The $K_{0.5}$ from the Hill equation is thus a mean apparent affinity which includes the mean affinity for the closed states, the mean affinity for the open states and the mean equilibrium constant for opening/closing. The fact that, in the presence of Ni^{2+} ions, P_o reaches 100% with cGMP but not with cAMP at saturation

of binding suggests that a channel protein with 4 cGMP bound is stabilized in the open conformation, while a channel with 4 cAMP bound oscillates between open and closed conformations. This indicates that the real affinity for cAMP is probably less than the $K_{0.5}$.

The dwell-time analyses carried out with the three nucleotides must be considered only as a comparative approach of the kinetic characteristics of channel activity rather than a kinetic analysis *"per se."* Indeed: (i) in order to increase the signal/noise ratio, the records were filtered at 300 Hz which allows reliable measurements of dwell times longer than 1 msec only (*see* Materials and Methods); (ii) in the presence of cGMP or 8Br-cGMP, the first current sublevel observed at low P_o corresponds to only 25% of the maximal current and is not well separated from the closed state (the situation is still worse in cAMP where no clear threshold between open and closed states was seen); therefore, the estimates of τ_c and τ_o are biased by the presence of those points which could not be reliably identified as belonging to the closed or open state; (iii) the basic closed/open scheme that was assumed for the analysis does not take into account the complexity of the model previously proposed (scheme *a*) and τ_c and τ_o are just mean transition rates.

With 8Br-cGMP or cGMP, both the dwell-time histograms and the power spectral densities reveal at least two time constants. By analogy with the results of Matthews and Watanabe (1988) in toad rods, the slower process could reflect the existence of bursts and the faster one could be associated with brief openings and closings within a burst. Matthews and Watanabe (1988) proposed that the duration of bursts, which depends on the nucleotide concentration, is related to different open conformations of channels. In scheme *a*, the opening of successive states as a function of nucleotide concentration could determine both the amplitude of the current and the duration of the bursts. While opening and closing within a burst have been shown to be independent of the cGMP concentration in toad rods (Matthews & Watanabe, 1988), in our experiments, the estimate of the fast components in the closed and in the open state cannot be achieved accurately enough to allow a confident analysis of their dependence upon nucleotide concentration.

With cAMP, neither duration histograms nor spectral densities reveal any slow component comparable to those measured in 8Br-cGMP or cGMP. The closed- and open-time distributions are described by single exponentials, with time constants between 2 and 3 msec for saturating cAMP. Both τ_c and τ_o seem to slightly depend on cAMP concentration. It is possible that the mean closed and open times measured in cAMP correspond to short-lived bursts, in comparison to those observed with cGMP, and that very fast transitions between the closed and open state could exist inside a burst, which would be completely undetectable under our experimental conditions. The short-lived bursts in cAMP could be explained, assuming that the channel opening is confined to the first step(s) of scheme *a*.

Potentiating Effect of Ni^{2+} Ions

The results presented here with Ni^{2+} ions added on the cytoplasmic side of the channel confirm those previously described (Ildefonse & Bennett, 1991). The potentiating effect illustrated with both cGMP and cAMP (and also observed with 8Br-cGMP, *not shown*) is related to a shift of $K_{0.5}$ (16 to 4 μM with cGMP, 740 to 225 μM with cAMP). With either cGMP or cAMP, the kinetic analysis confirms that the mean open time τ_o is largely increased and the mean closed time τ_c is reduced by the addition of Ni^{2+}. In the case of cGMP, however, when τ_c and τ_o are related to the open probability, no significative modification of their values is observed upon addition of Ni^{2+}. This result could indicate that the shift in $K_{0.5}$ is due to a modification by Ni^{2+} ions of the affinity of the channel sites for cGMP rather than to a direct effect on the kinetic constants between the closed and open states: the effect of Ni^{2+} ions could lead to a displacement from the first steps of the reaction, on the left, to the last ones, on the right in scheme *a*. When the channel is opened by cAMP, in the presence of Ni^{2+} ions, the kinetic parameters become quite comparable to those measured when the channel is opened by cGMP. In this case, a modification of the affinity for cAMP by Ni^{2+} ions cannot account alone for the effect observed, suggesting that Ni^{2+} may also modify the open/closed transition rates. Since four current levels can be observed in the presence of Ni^{2+}, these results also suggest that Ni^{2+} could favor cooperative binding of cAMP to the four sites of the channel. The apparent affinity of cAMP for the channel in the presence of Ni^{2+} still remains less than that for cGMP by a factor of about 50.

Combined Effects of cGMP and cAMP on Single-Channel Gating

The observation of both a potentiating and an inhibiting effect of cAMP on the cGMP-induced single-channel activity as a function of the concentration of cAMP confirms the hypothesis proposed by Furman and Tanaka (1989) that the same channel is involved

in the binding of the two nucleotides. The increase in open probability induced by addition of low cAMP concentrations is related to a shift of $K_{0.5}$ for cGMP from 12 to 6 μM, and, as observed when the channel is opened by cGMP + Ni^{2+}, the shift in $K_{0.5}$ can account for the kinetic modifications: related to the open probability, τ_c and τ_o have the same value in the presence of cGMP alone or of cGMP + low cAMP concentrations. For higher cAMP concentrations, however, these rates are faster, as illustrated in the experiment with 8Br-cGMP, leading to an apparent decrease in the current amplitude similar to that observed with cAMP alone. The efficient concentration of cAMP on potentiating the cGMP- or 8Br-cGMP-induced activity is very far below the threshold value estimated with cAMP alone, particularly in the presence of 8Br-cGMP, as previously reported by Filatov et al. (1989). This indicates that the real affinity for cAMP, at least for the first site, is much higher than the $K_{0.5}$, which, as pointed above, is only an apparent affinity. The model proposed by Furman and Tanaka (1989) to account for the effects of cAMP supposed the existence of three binding sites, the open conducting state being determined by two common ligands; their theoretical fit also required that the affinity for cAMP in the first step be higher than the $K_{0.5}$ measured from the dose-response curves (560 μM and 1.5 mM, respectively). The model of Furman and Tanaka (1989) is oversimplified when compared to the model proposed in scheme *a*, but a similar competitive binding of cAMP and cGMP to the nucleotide site can account for our results at the single-channel level: the apparent shift in $K_{0.5}$ for cGMP could be explained by occupancy of the first site (or of the first two ones) by a molecule of cAMP. The presence of a regulatory cAMP-binding site different from the cGMP-binding site seems to be excluded from the sequence which reveals the presence of a single nucleotide site per monomer (Kaupp et al., 1989).

Till now, no cAMP concentration measurements seem to have been performed in the rod, although results concerning measurements of a light-sensitive pool of cAMP in mouse retina suggest that most of it could be in photoreceptors (Cohen & Blazynski, 1987). The possibility that cAMP could modulate the cGMP-gated channel, which was suggested by Furman and Tanaka (1989) and by Filatov et al. (1989), still remains speculative.

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